the plant journal

SEB Society for Experimental Biology

The Plant Journal (2019) **98,** 346–358 doi: 10.1111/tpj.14223

Enhancing microRNA167A expression in seed decreases the α-linolenic acid content and increases seed size in *Camelina sativa*

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Received 25 October 2018; revised 11 December 2018; accepted 18 December 2018; published online 3 January 2019.

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SUMMARY

Despite well established roles of microRNAs in plant development, few aspects have been addressed to understand their effects in seeds especially on lipid metabolism. In this study, we showed that overexpressing microRNA167A (miR167OE) in camelina (*Camelina sativa*) under a seed-specific promoter changed fatty acid composition and increased seed size. Specifically, the miR167OE seeds had a lower α-linolenic acid with a concomitantly higher linoleic acid content than the wild-type. This decreased level of fatty acid desaturation corresponded to a decreased transcriptional expression of the camelina fatty acid desaturase3 (*CsFAD3*) in developing seeds. MiR167 targeted the transcription factor auxin response factor (CsARF8) in camelina, as had been reported previously in Arabidopsis. Chromatin immunoprecipitation experiments combined with transcriptome analysis indicated that CsARF8 bound to promoters of camelina *bZIP67* and *ABI3* genes. These transcription factors directly or through the ABI3-bZIP12 pathway regulate *CsFAD3* expression and affect α-linolenic acid accumulation. In addition, to decipher the miR167A-CsARF8 mediated transcriptional cascade for *CsFAD3* suppression, transcriptome analysis was conducted to implicate mechanisms that regulate seed size in camelina. Expression levels of many genes were altered in miR167OE, including orthologs that have previously been identified to affect seed size in other plants. Most notably, genes for seed coat development such as suberin and lignin biosynthesis were down-regulated. This study provides valuable insights into the regulatory mechanism of fatty acid metabolism and seed size determination, and suggests possible approaches to improve these important traits in camelina.

Keywords: microRNA167, transcription factors, *Camelina sativa*, fatty acid desaturase 3, α-linolenic acid, seed size.

INTRODUCTION

Vegetable oils (e.g., triacylglycerols, TAG) from oilseed crops are an important source of human nutrition and increasingly various industrial feedstocks such as biofuels (Lu *et al.*, 2011). However, the fatty acid composition of most seed oils is often not ideal for either food or industrial applications. For example, camelina (*Camelina sativa*) is a newly introduced crop in North America that is under development mainly for biofuel production (Shonnard *et al.*, 2010). One of the important limitations for camelina is the suboptimal quality of its oil (Ciubota-Rosie *et al.*, 2013). Conventional camelina oil is not ideal for use in biofuels because of its high content of undesirable fatty acids especially polyunsaturated fatty acids (50–60% of the total TAG) (Kang *et al.*, 2011), which are known to be associated with low oxidative stability of the biodiesel (Knothe, 2008). The enrichment of oleic acid (18:1; numbers of carbon:double-bond) in vegetable oils improve biodiesel properties while maintaining acceptable oxidative stability and cold temperature performance (Knothe, 2008). Conversely, the high content of the ω-3 α-linolenic acid (18:3) in camelina is desirable for food/feed applications due to its potential health benefits (Pilgeram *et al.*, 2007).

A comprehensive understanding of lipid metabolism is required to effectively improve fatty acid composition to

meet different needs. Many important genes controlling fatty acid modification have been characterized in Arabidopsis and some oilseed crops (Wallis and Browse, 2010). In oilseed, fatty acids are synthesized in the plastid, and are exported into the cytosol mainly in the form of the monounsaturated 18:1 along with smaller amounts of the saturated palmitic acid (16:0) and stearic acid (18:0). The 18:1 is further desaturated to form polyunsaturated linoleic (18:2) and α-linolenic (18:3) acids by the fatty acid desaturases FAD2 and FAD3 in the endoplasmic reticulum (ER) via phosphatidylcholine (PC) substrates, or elongated into eicosenoic (20:1) and erucic (22:1) acids by fatty acid elongase FAE1 (Li-Beisson *et al.*, 2013). These enzymes are effective targets to reduce polyunsaturates and very-long-chain fatty acids in camelina seed oil using RNA interference (Kang *et al.*, 2011; Nguyen *et al.*, 2013) or CRISPR/Cas9 genome editing techniques (Jiang *et al.*, 2017; Morineau *et al.*, 2017; Ozseyhan *et al.*, 2018). Several other biosynthetic enzymes such as phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), lysophosphatidylcholine acyltransferases (LPCAT), and diacylglycerol acyltransferases (DGAT) also affect the fatty acid composition of TAG by regulating the acyl flux through its precursors including PC and diacylglycerols (Lu *et al.*, 2009; Bates *et al.*, 2012; Marmon *et al.*, 2017).

Oil accumulation is an integral part of seed maturation, which is tightly controlled by genetic and biochemical factors such as transcription factors (TFs) and plant hormones (e.g. abscisic acid, gibberellin and auxin) (Santos-Mendoza *et al.*, 2008; Baud *et al.*, 2016). In *Arabidopsis thaliana*, the L-AFL TFs of the B3-domain proteins, including LEAFY COTYLEDON2 (LEC2), ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and the B subunits of the NF-Y family of trimeric TFs LEC1 (also called NF-YB9) and LEC1-LIKE (L1L, NF-YB6), act as central positive regulators during seed maturation (Santos-Mendoza *et al.*, 2008). Hormone signaling interacts with the L-AFL regulatory network during seed maturation (Gaj *et al.*, 2005; Nag *et al.*, 2005; Stone *et al.*, 2008; Liu *et al.*, 2013). For example, LEC2 induces the maturation phase by rapidly changing auxin responses (Stone *et al.*, 2008). Auxin and sucrose are required for LEC1 in the promotion of embryonic cell identity and division (Casson and Lindsey, 2006). Auxin also increases expression of FUS3 that regulates the synthesis of GA and ABA (Gazzarrini *et al.*, 2004), and induces auxin response factors (ARF10/16)-mediated *ABI3* activation for seed dormancy (Liu *et al.*, 2013).

The interplay of hormone-regulated gene expression governs seed development as well as metabolism of storage products (Baud and Lepiniec, 2010). TAG biosynthesis is at least partly regulated at the transcriptional level involving several regulatory factors including the L-AFL TFs (Baud and Lepiniec, 2010; Baud *et al.*, 2016). The LEC2 targets the WRINKLED1 (WRI1), an APETALA2-ethylene responsive element-binding protein (AP2-EREBP) family transcriptional factor, which directly regulates fatty acid biosynthesis (Cernac and Benning, 2004; Baud *et al.*, 2007; Kanai *et al.*, 2016). Mutants of the central TFs (e.g., *lec1*, *fus3*, and *wri1*) accumulate reduced oil (Meinke *et al.*, 1994; Cernac and Benning, 2004), while overexpression of these TFs increases seed oil content (Liu *et al.*, 2010; Shen *et al.*, 2010; Zhang *et al.*, 2016; Zhu *et al.*, 2018). The composition of fatty acids in storage oil may also be influenced by regulatory events during seed maturation. ABA or osmotic conditions in maturing seed influence the distribution of fatty acids between the pathways leading to desaturation or elongation (Finkelstein and Somerville, 1989, 1990). An Arabidopsis mutant of the *bZIP67*, which encodes a basic leucine zipper domain TF that activates *FAD3* downstream of LEC1 and FUS3, contained decreased 18:3 in seeds (Mendes *et al.*, 2013). Previous studies have suggested links between auxin and the fatty acid desaturation efficiency in soybean embryos (Liu *et al.*, 1995), and auxin induced the expression of the *FAD3* gene in Arabidopsis roots (Matsuda *et al.*, 2001). Nonetheless, in contrast with the control of oil quantity, the transcriptional regulation on key genes that determine seed fatty acid composition is less well understood.

MicroRNAs control gene expression by post-transcriptional regulation, and therefore play key roles in many developmental aspects including fruit growth and seed development (Zhang *et al.*, 2013a; José Ripoll *et al.*, 2015). For example, microRNAs regulate the timing of embryo maturation in Arabidopsis in part by repressing master regulators such as LEC2 and FUS3 (Willmann *et al.*, 2011). Manipulating microRNA levels to disrupt the transcriptional homeostasis of their target genes therefore provides a powerful tool to decipher regulatory mechanisms of developmental and metabolic programs. We have previously profiled miRNAs in camelina. Several miRNAs that are highly expressed showed differential expression (DE) between leaves and developing seeds, suggesting their potential roles in seed development and lipid metabolism (Poudel *et al.*, 2015). Among those, miR167 has been previously shown in Arabidopsis and tomato to target the auxin response factors (ARF6 and ARF8), which regulate plant reproduction such as flower and fruit development (Goetz *et al.*, 2006; Wu *et al.*, 2006; Liu *et al.*, 2014). Interestingly, two fatty acid desaturases (*ADS4.1* and *ADS4.2*) were among the genes under-expressed during flower maturation in the Arabidopsis *arf6*/*arf8* mutant (Reeves *et al.*, 2012). We hypothesized that miR167 might be involved in regulating fatty acid metabolism and seed development in camelina. Here we show that seed-specific overexpression of miR167A in camelina causes decreased content of α-linolenic acid (18:3) in the oil and increased seed size.

RESULTS

Effects of seed-specific overexpression of miR167A in camelina

To study the function of miR167 in seed, a construct containing miR167A under the control of a seed-specific

phaseolin promoter was transformed into camelina (cv. Suneson). We obtained 12 transgenic T1 (miR167OE) plants selected by herbicide resistance conferred by the *bar* gene (Snapp *et al.*, 2014). To obtain an initial evaluation of the effect of miR167A on fatty acid metabolism, 12 seeds from each T1 plant were individually analyzed by high-throughput gas chromatography (GC) (Lu *et al.*, 2006). Samples of these seeds were presumably wild-type or hemizygous for the *miR167A* transformation events. Fatty acid composition of single seeds varied, in some seeds α-linolenic acid (18:3) content decreased and linoleic acid (18:2) was increased compared with the wild-type (Figure S1). This result suggested possible effects of miR167A overexpression on seed lipid metabolism and encouraged us to investigate its mechanism. All transgenic lines were then advanced to the next generations and, consequently, three homozygous T3 lines were chosen for further characterization. The insertion and overexpression of miR167A were confirmed by genomic and quantitative RT-PCR (Figure S2). Two independent miR167OE lines (#4, #10) showing high miR167A expression were chosen for further studies.

The changed fatty acid composition was confirmed in homozygous miR167OE T3 seeds. In mature seeds, the levels of 18:2 and 18:3 in miR167OE lines were 50% increased and 43% decreased, respectively, compared with seeds from Suneson, while other FAs remained largely unchanged (Figure 1a). During seed development, 18:2 reached at the highest level at 12 days after flowering (DAF) in Suneson before gradually decreasing. In miR167OE seeds it remained higher throughout seed development, although decreased at a similar rate after 12 DAF compared with Suneson (Figure S3a). Concurrently, 18:3 gradually increased in Suneson as seeds matured, but in miR167OE its levels did not change significantly and started to fall below that of Suneson after 12 DAF so that 18:3 reached only to about half of its amount in Suneson at 24 DAF (Figure S3b). This result clearly indicated that the conversion of 18:2 to 18:3 had been compromised in the miR167OE developing seeds.

Compared with Suneson, the miR167OE lines also had increased seed size and seed weight. Mature dry seeds of miR167OE had about a 33% increase in seed size and a 56% increase in seed weight compared with Suneson in the greenhouse conditions (Table 1). However, based on seed dry weight, the percentages of oil, protein, and soluble sugar contents did not change significantly (Table 1). A closer examination of pods and seeds during fruit and seed development revealed that miR167OE lines had slightly delayed seed maturation (2–4 days) compared with the wildtype plants grown together in the greenhouse (Figure 1b).

RNA-seq analysis suggests miR167A-mediated pathways that affect lipid metabolism and seed size

To reveal miR167A pathways that might determine the fatty acid profile and seed size in camelina, we performed

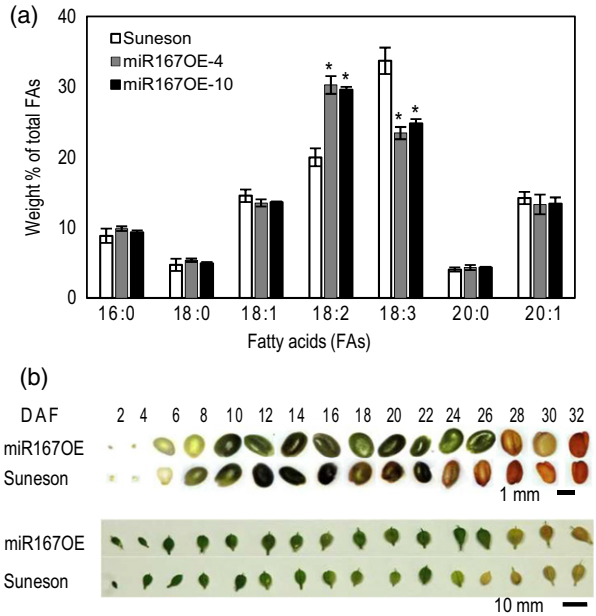

Figure 1. The effects of miR167A overexpression in camelina seeds.
(a) Fatty acid composition was determined in wild-type control (Suneson, white bars) and two independent T3 homozygous miRNA167A overexpressing transgenic lines (miR167OE, black bars). Data are the average with SE (error bars) of three replicas. Asterisks above the bars indicates significant differences (one-way analysis of variance).
(b) Seed and pod sizes change during seed development at 2–32 days after flowering (DAF) in Suneson and miR167OE.

RNA sequencing (RNA-seq) using Suneson and miR167OE developing seeds at 8, 10, and 12 DAF. Camelina seeds at these stages are actively growing and accumulating storage products (Rodríguez-Rodríguez *et al.*, 2013). Comparison of read counts by principal component analysis indicated good reproducibility of biological replicate RNA-seq libraries and overall differences between Suneson and miR167OE samples (Figure S4a). We performed BLAST searches against the Arabidopsis database at The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/). Multiple homologs in the hexaploidy *C. sativa* genome were found for each ortholog in *A. thaliana* (Kagale *et al.*, 2014). Nearly 86% (81 005 of 94 495) of camelina genes had at least one homolog that matched *A. thaliana* genes, and the closest homolog was used to assign putative GO terms to camelina genes. Our analyses identified 5391 camelina genes that have overall DE levels between Suneson and miR167OE (Figure S4b). When looking specifically at time-related DE, we found 3274 and 433 DE genes using DESeq2 and spliceTimeR, respectively (Table S1). The most enriched GO terms of DE genes (Figure S5) include those related to seed oil metabolic processes (GO:0006629, GO:0010344), seed development, and seed maturation (GO:0048316, GO:0010431). Interestingly, several highly enriched GO terms are related to

Table 1 Comparison of seed characteristics in Suneson and miR167OE

	Pod size (mm^2)	Seed size (mm^2)	Seed weight (mg/100 seeds)	Oil content (%)	Protein content (mg g^{-1} DW)	Sugar content (mg g^{-1} DW)
Suneson	20.5 ± 2.4^a	1.2 ± 0.1^a	83.5 ± 12.7^a	32.5 ± 3.2^a	148.7 ± 11.3^a	55.1 ± 8.4^a
miR167OE	29.4 ± 0.7^b	1.6 ± 0.2^b	130.0 ± 25.9^b	32.6 ± 1.5^a	147.1 ± 12.7^a	55.3 ± 4.1^a

Values are average ± SD on measurements on seeds from individual plants (n = 4–15) of each genotype grown in controlled conditions. Different letters above the values indicate significant differences (one-way analysis of variance). DW, dry weight.

phenylpropanoid metabolic process (GO:0009698), including suberin biosynthetic process (GO:0010345), cutin biosynthetic process (GO:0010143), and lignin biosynthesis (GO:0009809), which are involved in seed coat development. Enrichment of some GO terms related to defense responses and programed cell death (PCD) suggested the roles of PCD in seed development (Sreenivasulu and Wobus, 2013). We confirmed the expression levels of some DE genes between miR167OE and Suneson using semi-quantitative RT-PCR (Figure S6).

For DE genes involved in lipid metabolism, all *FAD3* genes (*CsFAD3; Csa05g033930, Csa07g013360, Csa16g014970*) were down-regulated by more than two-fold in miR167OE, which agreed with its reduced 18:3 content compared with the wild-type. Most of other TAG biosynthesis genes remained unchanged, except that *PDAT1* was increased in miR167OE (Figure 2a, Table S2). Among the L-AFL TFs that control seed maturation and lipid synthesis, *ABI3* and *FUS3* were decreased, however *LEC1* and *LEC2* were increased in miR167OE. The *bZIP67*, which has been shown to regulate *FAD3* in Arabidopsis (Mendes *et al.*, 2013), was down-regulated. *WRI1*, which acts downstream of *LEC2* and directly regulates fatty acid synthesis genes, was not found in the DE gene lists. In contrast with limited changes of storage lipid biosynthesis genes, several genes involved in cuticular lipids were affected. The TFs MYB9 and MYB107 regulate the seed coat polymer suberin biosynthesis (Lashbrooke *et al.*, 2016). Their down-regulated gene expression in miR167OE coincided with reduced transcripts of several genes for long chain fatty acid metabolism (*LACS2* (Schnurr and Shockey, 2004), *FAR4, 5* (Domergue *et al.*, 2010), *KCS20* (Lee *et al.*, 2009)), acyltransferases *GPAT4,5,6* (Beisson *et al.*, 2007; Li *et al.*, 2007; Petit *et al.*, 2016), *CYP86A2*, *B1* (Compagnon *et al.*, 2009), and *LPT1* (Deeken *et al.*, 2016) that are required for suberin biosynthesis and deposition in seed coat (Figure 2f). Besides lipid metabolism, genes expressed at later stages of embryogenesis including those encoding oleosins (*OLE1-4*), which are associated with oil-body biogenesis, seed storage proteins (cruciferins and albumins), and late embryogenesis abundant proteins (Figure 2c, Table S2) were down-regulated.

A few genes involved in cell division (*CDC20.1*, *CDC48C*, *CDKB2;1*, and *CYC2*) that showed increased expression might have contributed to increased seed size in miR167OE (Table S2). Analyzing seed transcriptomes indicated that several genes whose orthologs have been shown to regulate seed size (Orozco-Arroyo *et al.*, 2015; Li and Li, 2016) had altered expression in miR167OE (Figure 2b, Table S2). Hormone response genes (*SAR1*, *BZR1*, and *CKX2*) were up-regulated while the *AXR2/IAA7* decreased. *MET1* and *FIS2* were up-regulated but *CYP78A6/A9* were greatly down-regulated. *MYB56,* a positive TF of seed size regulator in Arabidopsis by controlling cell wall metabolism (Zhang *et al.*, 2013b), was up-regulated in miR167OE compared with Suneson at all stages. As seed development is also under metabolic regulation (Borisjuk *et al.*, 2008), we also examined genes involved in metabolism of sugar and other seed storage compounds. Several genes involved in sucrose metabolism and transport (e.g., *SUS2*, *SUS3*, *SUT1*, and *SPS3F*) decreased (Figure 2d). The amylases (*AMY2*, *B-AMY*) were greatly increased along with the *GA3OX3* for gibberellin biosynthesis, suggesting enhanced starch mobilization associated with the PCD during seed maturation (Sreenivasulu and Wobus, 2013). Interestingly, the expression of a protein kinase *SnRK2.6* was up-regulated. In Arabidopsis, this gene regulates sucrose homeostasis and its overexpression decreases trienoic fatty acids (16:3 and 18:3) in leaves (Zheng *et al.*, 2010). In the flavonoid biosynthesis pathway, genes of regulators (*MYB115*, *MYB5*, *GL2*, *ANL2*) and enzymes (*CHI*, *DFR*, *ANS*, *BAN*) of the proanthocyanidin synthesis (Xu *et al.*, 2014; Wang *et al.*, 2017) were up-regulated, while the branches of flavonol synthesis (*MYB111*, *FLS3,5*) (Stracke *et al.*, 2007) and lignin biosynthesis (*MYB58*, *PAL4*, *4CL1*, and *CCR*) (Zhou *et al.*, 2009) were down-regulated (Figure 2e; Table S2).

MiR167A targets CsARF8 in camelina

It has been shown in Arabidopsis that miR167 targets the auxin response factors (ARF6 and ARF8) to regulate gene expression (Wu *et al.*, 2006). To test whether this also occurs in camelina, we examined the gene expression levels of putative *CsARF6* and *CsARF8* genes in developing seeds. By RNA-seq analysis, we found three transcripts for each (Csa03g033520, Csa14g037990, Csa17g043290; and Csa10g040610, Csa11g050750, Csa12g074460) that matched to the Arabidopsis *ARF6* and *ARF8* genes,

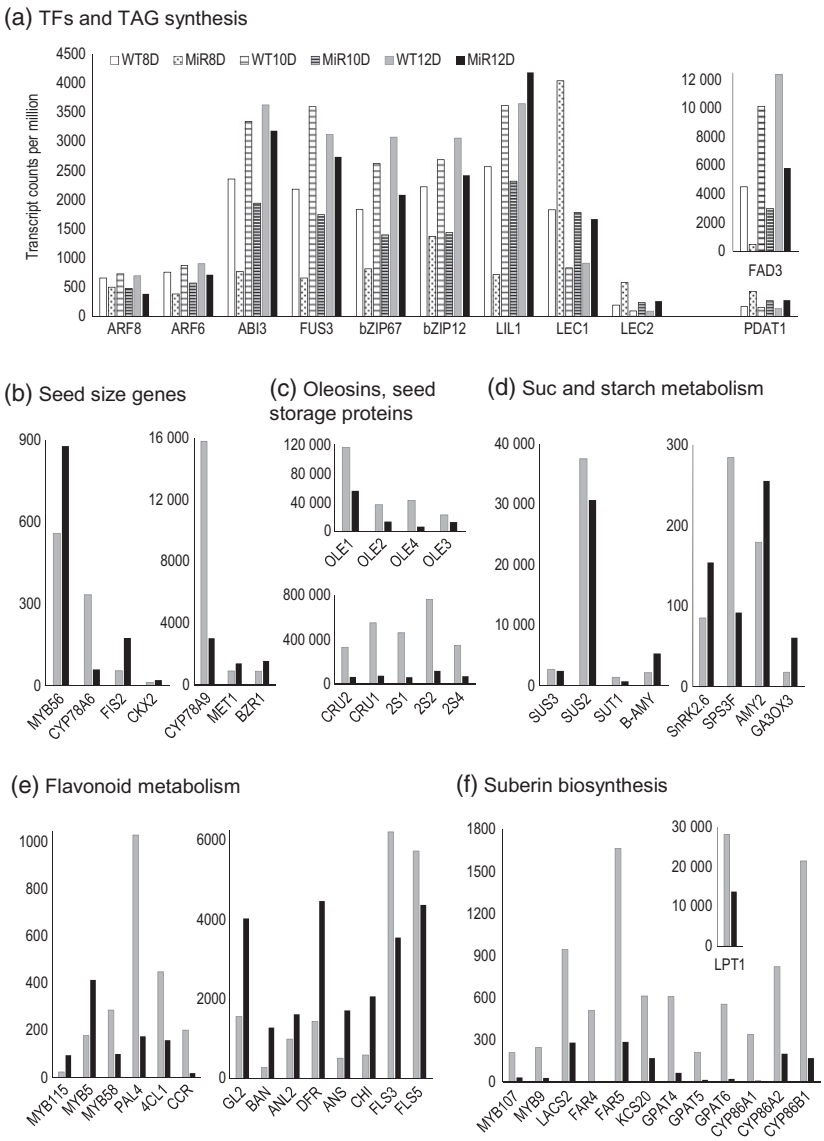

Figure 2. Comparison of gene transcript levels in Suneson and miR167OE during seed development. Values are totals of all homologs that represent averages of three replicas. For simplicity, only data at 12 DAF were used in B-F to illustrate the changed gene expression levels as they all had similar trends at 8, 10, and 12 DAF. Data for all stages and details of gene names and descriptions are listed in Table S2.

respectively. Expression levels of these putative *CsARF6* and *CsARF8* genes varied (Table S2), but overall, they significantly decreased compared with Suneson at the same developmental stages (Figure 2). These results suggested that miR167A might regulate *CsARF6* and *CsARF8* in camelina in a similar manner as shown in other dicots including Arabidopsis (Wu *et al.*, 2006) and Tomato (Liu *et al.*, 2014).

To confirm that miR167A targeted *CsARF6* and *CsARF8* in camelina, we transformed a modified target gene in miR167OE for seed-specific expression. We chose *CsARF8* as it appeared to be more profoundly down-regulated in miR167OE when seeds are actively synthesizing storage lipids (12 DAF), and because ARF8 and ARF6 have partially overlapping functions (Liu *et al.*, 2014). We designed the *mCsARF8* sequence that contained 10 nucleotide mutations at the miR167 target site on the C-terminus of *CsARF8* without causing changes in amino acid coding sequences. The predicted base pairs per 21 target nucleotides between miR167 versus CsARF8 and miR167 versus mCsARF8 were 18 nucleotides and 8 nucleotides, respectively (Figure 3a). Transgenic miR167OE-mCsARF8 plants were obtained by the DsRed selection marker and six T2 lines were analyzed for their seed traits. As shown in Figure 3b, compared with the brown miR167OE seeds, the FA composition of the red seeds was similar to wildtype levels. Seed size and seed weight were also partially reduced although still larger than Suneson (Figure S7). No other obvious changes were noticed in the miR167OE-mCsARF8 plants compared with miR167OE and Suneson plants grown in the greenhouse. Taken together, we concluded that miR167A targeted CsARF8 and caused decreased 18:3 accumulation and also involved in seed size enlargement in miR167OE seeds.

(a)

```
miRNA167      21    AUCUAGUACGACCGUCGAAGU  1
                    ::::::: ::::::::::::
CsARF8 (1)    2273  UAGAUCAGGCUGGCAGCUUGU  2294
CsARF8 (2)    2273  UAGAUCAGGCUGGCAGCUUGU  2294
CsARF8 (3)    2270  UAGAUCAGGCUGGCAGCUUGU  2291
                      R  S  G  W  Q  L

miRNA167      21    AUCUAGUACGACCGUCGAAGU  1
                      :      : :::::  :
mCsARF8       2273  GCGGAGCGGGUGGCAAUUAGU  2293
                      R  S  G  W  Q  L
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(b)

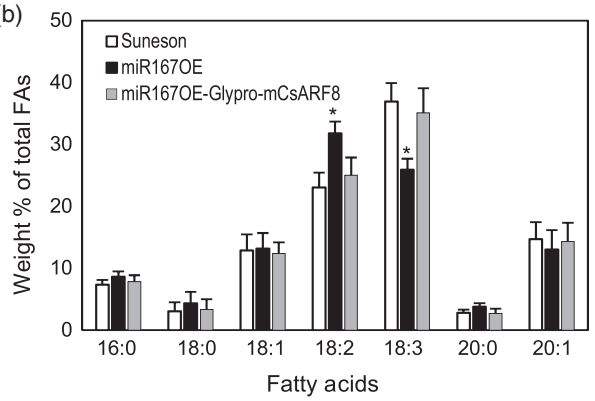

Figure 3. Effect of overexpressing mutated target site of CsARF8 (mCsARF8) on seed fatty acid composition.
(a) Sequences of miRNA167 target site on three CsARF8 mRNAs, and mutated target site of mCsARF8. Amino acids sequences are shown below.
(b) FA composition of seeds expressing mutated CsARF8 in the background of miR167OE plants. Data are the average ± SE (error bars) of three replicas. Asterisks above the bars represent significant differences.

MiR167A suppresses *CsFAD3* expression through the L-AFL regulatory network

Our results suggested that miR167A might regulate *CsFAD3* expression through CsARF8. ARF8 is known as a transcriptional activator, which binds with specificity to auxin-response elements (TGTCTC, AuxRE) in auxin-responsive promoters of primary/early genes (Ulmasov *et al.*, 1999). The *CsFAD3* gene promoters do not contain AuxRE sequences, suggesting the unlikelihood of direct regulation of *CsFAD3* by CsARF8. To search for additional TFs, we performed chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) experiments. Candidates were chosen from those significant DE genes that contained putative *cis* elements in their promoter regions within 2 kb (−2000 to −1) upstream of the transcriptional start site. Since most DE gene homologs had similar changes in expression, we chose one homolog for each gene that had the highest expression levels in all ChIP assays.

We found three homologs for each camelina *ABI3* and *bZIP67* genes, all showed decreased transcripts in miR167OE (Table S2). Putative AuxREs were identified in all six promoter sequences. We chose one homolog for each, *Csa15g050420* (*CsABI3*) and *Csa09g034290* (*CsbZIP67*), which had two putative AuxREs at −508 and −1052, and two putative AuxREs at −238 and −839, respectively (Figure S8). Two down-regulated genes, *LEA4-2* and *CsbZIP12*, the latter contains a putative AuxRE at +224, were also included in the experiment. Our results showed that CsARF8 bound to chromatin fragments associated with the *CsABI3* and *CsbZIP67* genomic DNA (Figure 4a). The binding of CsARF8 to both promoters was specific because we could not detect any immunoprecipitated fragments from *CsFAD3* and other genes such as *CsbZIP12* and *CsLEA4* (Figure 4a). These results suggested that CsARF8 controls *CsFAD3* expression through CsABI3 and CsbZIP67.

Similar approaches were applied to determine downstream TF−promoter interactions by ChIP-PCR. Previously, it has been demonstrated in Arabidopsis that bZIP67 directly regulates *FAD3* by binding to its promoter at the ABA response element (ABRE) core ACGT (or G-box) (Mendes *et al.*, 2013). The *CsFAD3* promoter contains three putative G-box sequences (Figure S8). Our ChIP-PCR analysis showed that CsbZIP67 bound to the *CsFAD3* (Csa07g013360) promoter at G-box 2 located between −372 and −376 bp upstream of the transcriptional start site (Figure 4c), indicating that CsbZIP67 also directly regulates *CsFAD3* expression in camelina. ABI3 is known as a positive regulator in seed maturation (Giraudat *et al.*, 1992). The Arabidopsis *abi3* mutant showed decreased 18:3/18:2 ratio in seed fatty acid composition (Finkelstein and Somerville, 1990), suggesting its involvement in *FAD3* gene regulation. ABI3 encodes a plant specific B3 domain TF that binds to the gene promoter RY motif (CATGCA) (Suzuki *et al.*, 1997; Reidt *et al.*, 2000; Braybrook *et al.*, 2006; Park *et al.*, 2011). This *cis* element is absent in the *CsFAD3* promoters, however we found a putative one at −387 in the promoter of the *CsbZIP12* (Csa05g011590) and two putative RY motifs at −33 and −480 in the promoter of *CsbZIP67* (Csa07g013360). ChIP-PCR results showed that CsABI3 bound to the *CsbZIP12* and *CsbZIP67* promoters (Figure 4b). This binding was four-fold higher than to the *SOMNUS* promoter, which was used as a positive control (Park *et al.*, 2011). No binding occurred between CsABI3 and *CsLEA4*, whose promoter contains one putative RY motif at −187 bp. These results thus strongly suggested that CsABI3 regulates *CsbZIP12* and *CsbZIP67* expression. More ChIP-PCR analysis indicated that CsbZIP12 directly bound to G-box2 and G-box3 in the *CsFAD3* promoters (Figure 4d).

To confirm that CsARF8 regulates *CsFAD3* expression through CsbZIP67 and CsABI3 – CsbZIP12, we cloned 1 kb region of the putative *CsFAD3* promoter in front of the β-glucuronidase (GUS) gene (pCsFAD3:GUS), and

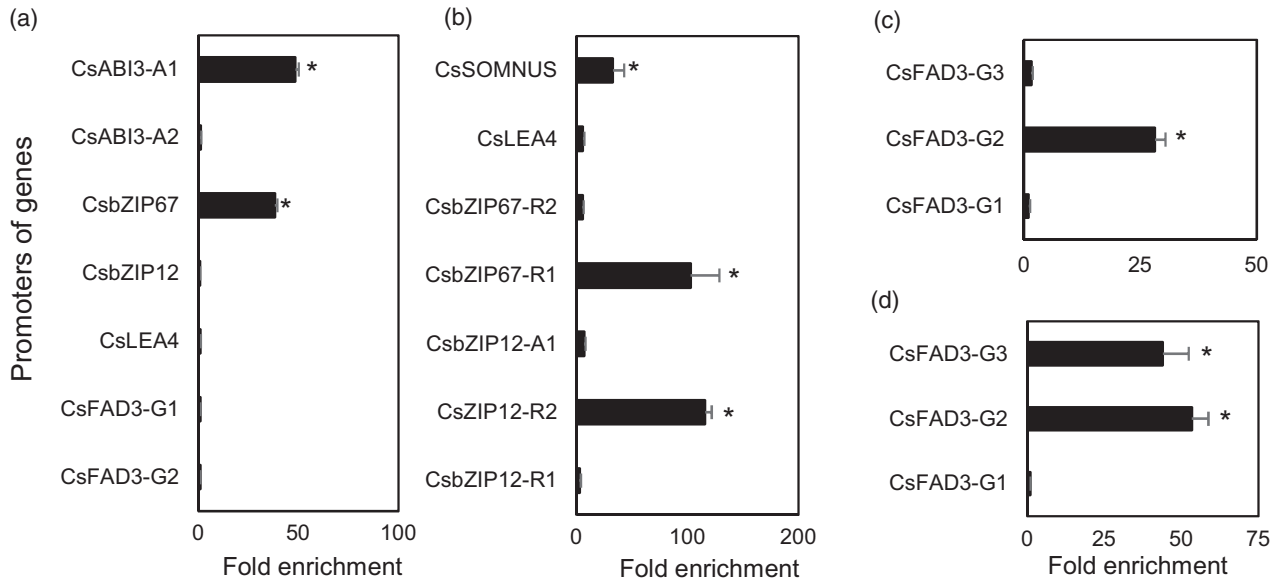

Figure 4. Determination of candidate gene promoters interacting with transcription factors using chromatin immunoprecipitation (ChIP). (a) CsARF8, (b) CsABI3, (c) CsbZIP67, and (d) CsbZIP12. Values are the mean with SE of three independent measurements and are normalized relative to lowest measurement of each ChIP experiment. Asterisk denotes a significant difference from the control.

transformed it into Suneson wild-type plants. Mesophyll protoplasts were prepared from pCsFAD3:GUS plants, and the same amount of *CsFAR8*, *CsABI3*, *CsbZIP67*, and *CsbZIP12* plasmids were transfected into the same number of mesophyll protoplasts. Compared with GUS activity from negative control samples containing pCsFAD3:GUS only, GUS activities from TF-transfected protoplasts were two- to three-fold enhanced (Figure 5). Therefore, we concluded that the expression of *CsFAD3* is regulated by miR167A-mediated gene regulation involving several TFs including CsARF8, CsbZIP67, CsABI3, and CsbZIP12.

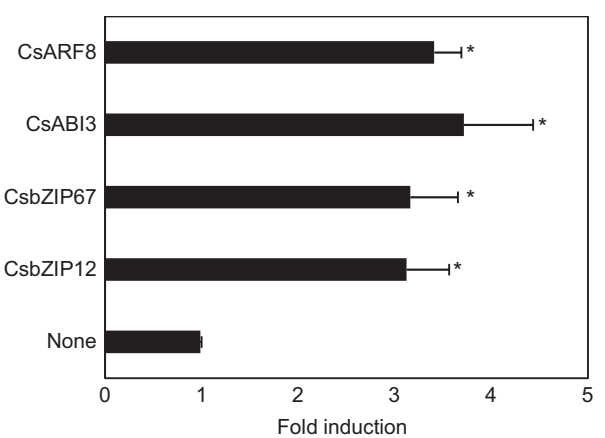

Figure 5. Effects of CsARF8, CsABI3, CsbZIP67, and CsbZIP12 on *CsFAD3* expression in the protoplast from *FAD3* promoter::GUS transgenic plants. Values are the mean with SE of three independent measurements and are normalized relative to none control sample of each GUS experiment. Asterisk denotes a significant difference from the control.

DISCUSSION

Storage oil biosynthesis is associated with seed maturation, and therefore is subject to regulation by numerous developmental, metabolic, and environmental factors (Santos-Mendoza *et al.*, 2008; Baud and Lepiniec, 2010; Kanai *et al.*, 2016). Besides internal signals such as plant hormones, the roles of microRNAs in regulating multiple developmental and physiological processes in plants including developing seed are well established (Nodine and Bartel, 2010; Willmann *et al.*, 2011). Here, we demonstrate that seed-specific overexpression of the miR167A in camelina caused an altered fatty acid composition in TAG, therefore revealing the importance of miRNAs in regulating plant lipid metabolism.

Camelina seed contains a high level of polyunsaturated α-linolenic acid (Kang *et al.*, 2011). The fatty acid desaturase FAD3 is primarily responsible for the production of 18:3 in seeds (Arondel *et al.*, 1992). In Arabidopsis, expression levels of *FAD3* corresponded to 18:3 accumulation (Shah and Xin, 1997), and a mutant with the *FAD3* gene duplication contained increased 18:3 in seeds (O'Neill *et al.*, 2011). The hexaploidy camelina has three alleles of *CsFAD3*, which may explain the higher levels of 18:3 (~35% versus ~20%) than found in the diploid Arabidopsis. However, there is limited information on transcriptional regulation of *CsFAD3* in camelina. Previously it has been shown that in Arabidopsis *FAD3* expression may be subject to various regulatory mechanisms including ones at the transcriptional level controlled by epigenetics (Wang *et al.*, 2016) and TFs (Mendes *et al.*, 2013). Here we demonstrate

that microRNAs, which act as post-transcriptional gene regulators of suites of genes, can be used to decipher a transcriptional mechanism that controls *CsFAD3* in camelina. Seed-specific overexpression of miR167A caused a decreased 18:3 content that corresponded to reduced *CsFAD3* transcripts. Previous studies in Arabidopsis indicated that miR167 targets the AUXIN RESPONSE FACTORs, ARF6 and ARF8 (Wu *et al.*, 2006). Our results suggested that miR167A also targeted their camelina orthologs, as *CsARF8* and *CsARF6* transcripts were significantly decreased in the miR167A-overexpressor compared with the wild-type. This was further supported by that mutations on the *CsARF8* targeting sequence cancelled the repressive effect of miR167A overexpression on 18:3 reduction. We therefore hypothesized that *CsFAD3* might be subject to regulation by the miR167A-CsARF8 mediated pathways. Transcriptome analysis and ChIP-PCR assays proved that CsARF8 bound to promoters of TFs *CsABI3* and *CsbZIP67*, both had decreased transcripts coincided with lower *CsARF8* expression. Further studies indicated that CsbZIP67 directly bound promoters of *CsFAD3*, while CsABI3 regulated *CsFAD3* through CsbZIP67 and another TF, CsbZIP12. All these TFs were able to activate *CsFAD3* gene expression (Figure 5). It has been shown previously in Arabidopsis that bZIP67 activated *FAD3* expression by binding to G-boxes in its promoter, and the *bzip67* mutant seed contained lower 18:3 (Mendes *et al.*, 2013). Taken together, a transcriptional regulatory cascade for *CsFAD3* expression is proposed in Figure 6. Accordingly, overexpression of miR167A suppressed *CsARF8*, which in turn deceased expression levels of *CsbZIP67*, *CsABI3*, and *CsbZIP12*, and therefore resulted in suppressed *CsFAD3* expression and lower levels of the 18:3 accumulation in the miR167OE seeds.

MiR167A overexpression also caused increased seed size and slightly delayed seed maturation process in camelina. As the L-AFL (LEC2/ABI3/FUS3, LEC1, and L1L) TFs act as central regulators of seed development, it is not surprising that the maturation process was affected by their altered expression levels in miR167OE. *LEC1* and *LEC2* have the highest mRNA levels during early stages while *FUS3* and *ABI3* are more strongly expressed during later stages of embryogenesis in Arabidopsis (Boulard *et al.*, 2017). The decreased expression of *CsABI3* and *CsFUS3* may primarily explain the effects of miR167 overexpression since phaseolin, a seed maturation stage-specific promoter (Chandrasekharan *et al.*, 2003), was used to drive miR167A in this study. It is unclear how *LEC1* and *LEC2* had higher levels of transcripts in miR167OE, though might have resulted from the interactions of the L-AFL TFs to maintain transcriptional homeostasis during seed development (Boulard *et al.*, 2017). FUS3 and LEC2 both target WRI1 to regulate fatty acid biosynthesis (Baud *et al.*, 2007; Wang and Perry, 2013). The unchanged *CsWRI1* expression

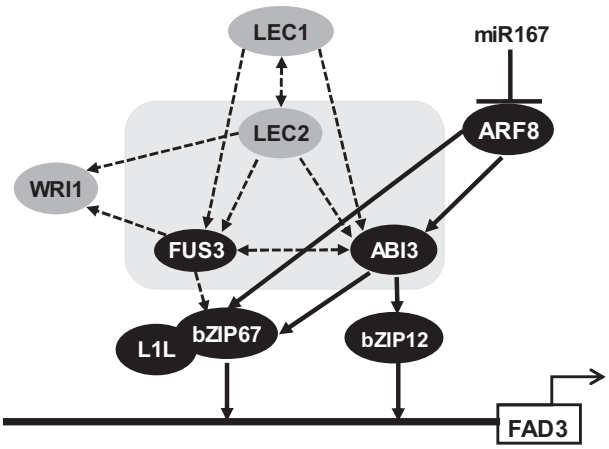

Figure 6. A model illustrating the miRNA167-ARF8 signaling pathways (solid arrows) interact with the central regulators (shaded area) of seed maturation and control *CsFAD3* expression. Transcription factors shown in black are down-regulated by miR167 overexpression. Dashed arrows are derived from Boulard *et al.* 2017; and Wang and Perry 2013.

level might have been the result of decrease in *CsFUS3* and increase in the relatively less abundant *CsLEC2*. Consequently, most of fatty acid and TAG synthesis genes controlled by CsWRI1 were not significantly changed. A delayed seed maturation in miR167OE agreed with down-regulated genes for oleosin and storage proteins (cruciferin and 2S albumin), which are known to be regulated by ABI3 and FUS3 (Nambara *et al.*, 1992; Wang and Perry, 2013; Baud *et al.*, 2016). This delay might have allowed the miR167OE seeds to accumulate similar levels of the final oil and protein contents compared with the wild-type (Table 1).

Several ARFs have been shown to regulate seed size. ARF2 is a repressor of cell division and organ growth, the Arabidopsis *arf2* (*megaintegumenta*) mutant has enlarged seed size (Schruff *et al.*, 2006). An *ARF18* mutation was found to increase seed weight and silique length in rapeseed, possibly by affecting cell wall development (Liu *et al.*, 2015). In *Jatropha curcas*, auxin treatment increases seed size, possibly by up-regulating expression of an *ARF19* homolog (Sun *et al.*, 2017). Our results suggested that ARF6/8 may also regulate seed size in camelina. Alteration of the CsARF8 target site of miR167 only partially recovered the seed size phenotype in miR167OE (Figure S7), suggesting that both CsARF8 and CsARF6 may be involved in regulating seed size although these two TFs are somewhat functionally redundant.

Seed size is determined by complex gene networks controlling growth and development of seed coat (maternal), embryo, and endosperm (zygotic) tissues. In Arabidopsis, larger seed size is primarily accounted for by the growth of seed coat and endosperm (Alonso-Blanco *et al.*, 1999). In our transcriptome analysis, several genes that were

orthologous to those previously identified that control seed size (Sreenivasulu and Wobus, 2013; Orozco-Arroyo *et al.*, 2015; Li and Li, 2016) had altered expression levels in the miR167OE (Table S2). These genes are involved in multiple pathways such as phytohormone responses (*BZR1*, *CKX2*) (Jiang *et al.*, 2013; Li *et al.*, 2013), epigenetic control in the endosperm (*MET1*, *FIS2*) (Köhler and Makarevich, 2006), seed coat flavonoid biosynthesis (*MYB115, CHI*, *DFR*, *ANS, FLS)* (Doughty *et al.*, 2014), and sugar metabolism (*SPS3F*, *SUS*, *SUT*) (Borisjuk *et al.*, 2008) and starch mobilization (*AMY*s*, GA3OX3*) that may be associated with PCD (Sreenivasulu and Wobus, 2013). Interestingly, the affected genes encode several TFs (MYB56, MYB5, MYB9/MYB107, and MYB58) that are primarily involved in seed coat development and may regulate seed size. Especially, several genes in suberin and lignin biosynthesis pathways were significantly down-regulated (Figure 2, Table S2). Cuticular polymers deposit in seed coat after cell expansion (Molina *et al.*, 2008). The delayed suberization and lignification of seed coat would allow for extended cell expansion and therefore increase seed size. How the miR167-CsARF6/8 complex regulates these genes and pathways is not clear, however, some may be mediated by perturbed L-AFL transcriptional networks. For examples, the seed coat differentiation and proanthocyanidin (PA) biosynthesis pathways (MYB115-DFR/CHI/ANS and MYB5-GL2/ANL2-BAN) (Gonzalez *et al.*, 2009; Wang *et al.*, 2017) were up-regulated. Previously, it has been shown that LEC2 targets *MYB118* (a homolog of *MYB115*) (Barthole *et al.*, 2014), and FUS3 functions through the epidermal regulator TRANSPARENT TESTA GLABRA1 (TGG1) (Tsuchiya *et al.*, 2004), which controls MYB5 and GL2 (Gonzalez *et al.*, 2009). We did not find transcripts for *CsMYB118*, but *CsMYB115* was up-regulated along with *LEC2*. *MYB5* and its downstream genes were all up-regulated, *TTG1* transcripts were also increased although not significant probably due to its repressive and positive regulation by FUS3 at different stages of embryogenesis (Tsuchiya *et al.*, 2004). In conclusion, our transcriptome analysis suggests that increased seed size in miR167OE is the results of combinatorial effects of multiple mechanisms. These possible regulatory pathways in camelina and their effects on seed size will need to be experimentally tested.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

A *Camelina sativa* cultivar 'Suneson' was used for all experiments. Camelina was grown in 9 cm pots filled with soil and Sunshine Mix (Clinton, OK, USA) (1:1) in the greenhouse. To harvest pods and seeds at 4–32 DAF, flowers were tagged using a thin slice of scotch tape around the pedicels. Samples for RNA extraction (8, 10, and 12 DAF) were harvested in liquid nitrogen and stored at −80°C until further analysis. For each sample, developing seeds were collected from three independent plants grown together at the same time points (DAF).

Measurement of seed storage products

To determine the fatty acid composition, the fatty acid methyl esters (FAMEs) were derived for gas chromatography using a Shimadzu 2010 GC fitted with a flame ion detector and a narrow-bore column (HP-Innowax 19091N-133; 30 m × 0.25 mm i.d. × 0.25 μm, 260°C max temperature; Agilent Technologies, Santa Clara, CA, USA) (Lu *et al.*, 2006). The oven temperature was programmed at 190°C initially followed by an increase of 20°C/min to 250°C and maintained for 8 min.

The oil content in seeds was measured using methods described previously (Snapp *et al.*, 2014).

For total protein measurement in seeds, total protein extraction from seed samples were prepared using methods described previously (Vigeolas *et al.*, 2007). The protein content was quantified using the bicinchoninic acid assay (Walker, 1994) with bovine serum albumin as the standard.

For soluble sugar measurement in seeds, we used the procedure (Li *et al.*, 2004). Ten microliter of extract was incubated with 1000 μl anthrone reagents (0.15% (w/v) anthrone, 72% (v/v) H_2SO_4, 28% (v/v) H_2O) at 100°C for 1 h. The absorbance was measured at 625 nm with glucose as the standard.

Gene expression analysis

Total RNA was isolated using Norgen miRNA extraction BioKit (Thorold, ON, Canada), measured by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). Nine libraries representing transcriptomes of Suneson and miR167OE at 8, 10, and 12 DAF were made and sequenced using standard Illumina RNA-seq protocols at the Joint Genome Institute (JGI). Two libraries were removed that were from different plants and different time points but clustered together. The other libraries clustered with libraries originated from the same plant and same time point.

We used three R packages to identify differentially expressed (DE) genes between Suneson and miR167OE: DESeq2 (Love *et al.*, 2014), maSigPro (Conesa and Nueda, 2016), and splineTimeR (Michna, 2016). With DESeq2, we used two approaches: (1) identifying global differences between the lines by using time as a covariate and (2) modeling the interaction term for genotype and time to focus on differential responses across time between the genotypes. For each method, we used 0.05 as the multiple testing-corrected *P*-value cutoff. For analysis using the 'maSigPro' and 'splineTimeR' packages, we used the 'edgeR' (Robinson *et al.*, 2010) R package to normalize read counts using the trimmed mean of *M*-values (TMM) method. Any genes for which no libraries had more than five reads were removed from DE analysis. The heatmap of DE genes was generated according to (Kolde, 2018).

To assign *Arabidopsis thaliana* orthologs to *Camelina sativa* genes, we used the program diamond (Buchfink *et al.*, 2015) to align *C. sativa* peptide sequences with *A. thaliana* sequences accessed from TAIR (Lamesch *et al.*, 2011). We used 'sensitive' mode and a query coverage cutoff of 50%, and we retained the *A. thaliana* gene with the top score. We assigned the same GO terms to *C. sativa* genes as their *A. thaliana* homologs. The R package 'topGO' (Alexa and Rahnenfuhrer, 2010) and Fisher's exact test were used to test for GO term enrichment in DE genes.

For the qRT-PCR or semi-qRT-PCR, the synthesis of first-strand cDNA was performed using SuperScript III reverse transcriptase (Waltham, MA, USA). The primer pairs used for the PCR listed in Table S3. For the qRT-PCR of miR167A, cDNAs were synthesized

using methods described previously (Varkonyi-Gasic *et al.*, 2007). Amplification of cDNA was carried out with the CFX96 Real-time system from Bio-Rad and PerfeCTa SYBR Green FastMix from Quantabio (Beverly, MA, USA) and the Livak method was used for calculating relative gene expression. The following standard thermal profile was used for PCR: 95°C for 5 min, 40 cycles of 95°C for 5 sec, 60°C for 10 sec, 72°C for 1 sec and melting curve analysis.

Transient expression and chromatin immunoprecipitation

For the transient expression and ChIP analysis, the coding regions of *CsARF8*, *CsABI3*, *CsbZIP67*, and *CsbZIP12* were amplified from the camelina cDNA, and PCR products were confirmed by sequencing and then inserted into the *Sal*I and *Xho*I or *Xba*I and *Bam*HI sites of the plasmid pBin35SRed2-sGFP to create chimeric green fluorescent protein (GFP) fusion constructs under the control of the *Cauliflower mosaic virus* 35S (CaMV 35S) promoter.

For β-glucuronidase (GUS, EC 3.2.1.31) activity measurement, camelina mesophyll protoplasts were prepared from transgenic lines harboring the promoter of CsFAD3:GUS as described previously (Yoo *et al.*, 2007). After polyethylene glycol-mediated transformation of sGFP fusion constructs (Jin *et al.*, 2001), protoplasts were incubated at 20°C for 16 h. GUS activities were determined using methods described previously (Aich *et al.*, 2001).

For ChIP assays, we used a modified procedure (Saleh *et al.*, 2008). In brief, camelina mesophyll protoplasts were prepared and transfected with 35S:CsARF8:GFP, 35S:CsABI3:GFP, 35S:CsbZIP67:GFP, or 35S:CsbZIP12:GFP. After 18 h incubation at 20°C, protoplasts were harvested and cross-linked in cross-linking buffer (0.4 M sucrose, 10 mM Tris-HCl pH 8, 1 mM PMSF, 1 mM EDTA, and 1% formaldehyde). Rabbit anti-GFP antibodies (Life Technologies, Eugene, OR, USA) and Dynabeads Protein G (Invitrogen, Carlsbad, CA, USA) were used to immunoprecipitate the genome fragments. PCRs were carried out with primer sets corresponding to *cis*-elements of target genes as listed in Table S3. All agarose gel images were analyzed using ImageJ software.

Plasmids and camelina transformation

To generate pGP-miR167A plasmids, camelina miR167A was synthesized by Genewiz, Inc. (South Plainfield, NJ, USA) and inserted into the vector pGlyPhas at *Bam*HI and *Xho*I sites in between phaseolin promoter and the glycine 3′ UTR (Lu *et al.*, 2006). The pGP-miR167A plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Camelina plants were transformed using vacuum-mediated floral dipping as described previously (Lu and Kang, 2008). Transformed plants was screened by spraying with glufosinate solutions as directed (Finale, Bayer CropScience, Research Triangle Park, NC, USA). The T2 lines that showed a approximately 75% survival rate indicating single insertions were selected. T3 seeds collected from the individual T2 plants were again selected with herbicide, and only lines showing 100% survival were selected as homozygous.

For pBinRed2-Gly (glycinin promoter):modified CsARF8 plasmids, 209 bp of C-terminus of CsARF8 was synthesized with the changing of nucleotides from TAGATCAGGCTGGCAGCTTGT to gcGgagcGGgTGgCAatTaGT by Genewiz, Inc. (South Plainfield, NJ, USA). The *Bbs*I- and *Xho*I-treated fragments of modified CsARF8 were ligated into pBin35SRed2 -CsARF8, which was linearized with *Bbs*I and *Xho*I. For pBinRed2-ProFAD3:GUS plasmids, the promoter region of CsFAD3 was amplified from camelina genomic DNA and inserted into a *Hin*dIII and *Sal*I-linearized pBinRed2 vector. Screening for the pBinRed2-Gly:mCsARF8 or pBinRed2-ProFAD3:GUS transformed plants was done by detecting red fluorescent seeds (Lu and Kang, 2008).

Statistical analysis

Statistical analyses were performed using Microsoft Office Excel 2016. One-way analysis of variance was used to test the equality of three or more means. Student's *t* tests were carried out to statistically compare pairs of means. Statistically significant differences were determined at a 5% level of probability for all comparisons.

ACKNOWLEDGEMENTS

We thank Drs. Kerri Barry, Natasha Brown, Chris Daum, Anna Lipzen and Yuko Yoshinaga at the U.S. Department of Energy Joint Genome Institute for technical assistance in RNA-seq experiments. This work was supported by the Office of Science (BER), US Department of Energy, grant no. DE-SC0016400, the US National Science Foundation, grant no. DBI-1339358, and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture Hatch project no. 1015638. The work conducted at JGI is supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Comparison of seed fatty acid composition in Suneson and miR167OE T1 plants.

Figure S2. Stable insertion of pGDP-miR167A plasmids and expression of miR167 in camelina.

Figure S3. Accumulation of 18:2 and 18:3 during camelina seed development.

Figure S4. PCA analysis of RNA-seq libraries and the heatmap of DE genes.

Figure S5. Enriched GO terms of DE genes.

Figure S6. Verification of RNA-seq results of selected genes by semi-qRT-PCR.

Figure S7. Seed traits of miR167OE expressing the modified CsARF8 target.

Figure S8. Schematic illustration of the position of putative *cis*-elements in gene promoters.

Table S1. Enrichment of GO terms of DE genes.

Table S2. Comparison of expression profiles of gene homologs in Suneson and miR167OE.

Table S3. List of primers used in this study.

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